Epigenetic regulation of profibrotic macrophages in systemic sclerosis-associated interstitial lung disease

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Abstract

Objectives: Systemic sclerosis-associated interstitial lung Disease (SSc-ILD) is the leading cause of death in patients with systemic sclerosis (SSc) with unclear pathogenesis and limited treatment options. Evidence strongly supports an important role for profibrotic, SPP1-expressing macrophages in SSc-ILD. We sought to define the transcriptome and chromatin structural changes of SPP1 SSc-ILD macrophages, so as to better understand their role in promoting fibrosis and to identify transcription factors associated with open chromatin driving their altered phenotype.

Methods: We performed single cell RNA-sequencing on 11 explanted SSc-ILD and healthy control lung samples, as well as single cell ATAC-sequencing on five lung samples to define altered chromatin accessibility of SPP1 macrophages. We predicted transcription factors regulating SPP1 macrophages using SCENIC and determined transcription factor binding sites associated with global alterations in SPP1 chromatin accessibility using Signac/Seurat.

Results: We identified distinct macrophages subpopulations by scRNA-seq analysis in normal and SSc-ILD lungs and determined the occurring gene expression changes during the change of normal macrophages into SPP1 macrophages. Analysis of open chromatin validated SCENIC predictions, indicating that MITF, TFEB, ATF6, SREBF1, BHLHE40, KLF6 ETV5 and/or members of the AP-1 family of transcription factors regulate SPP1 macrophage differentiation.

Conclusions: Our results shed light on the underlying changes in chromatin structure and transcription factor regulation of profibrotic SPP1 macrophages in SSc-ILD. Similar alterations

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in SPP1 macrophages may underpin fibrosis in other organs involved in SSc and point to novel targets for the treatment of SSc-ILD, targeting profibrotic macrophages.

INTRODUCTION

Systemic sclerosis (SSc) is a multisystem, autoimmune, fibrotic disease of unknown etiology with life-threatening fibrotic complications, such as SSc-associated interstitial lung disease (SSc-ILD) (1, 2). Despite advances in new SSc-ILD treatment options, such as nintedanib (3), tocilizumab (4, 5), and myeloablative autologous stem-cell transplantation (6), SSc-ILD remains difficult to treat. The consequences on survival and quality of life are important since approximately 50% of SSc patients develop SSc-ILD (2) and 33% of SSc patients die of SSc-ILD fibrotic complications (7).

Increasing evidence supports important roles for macrophages in SSc-ILD. Specifically, macrophage associated gene expression in lung biopsies correlates with progressive SSc-ILD, worsening lung fibrosis on high-resolution computed tomography (HRCT), and reduced performance on pulmonary function tests (PFTs) (8). SSc-ILD lungs show strongly upregulated expression of chemokine C-C motif ligand 18 (CCL18), which is expressed primarily by macrophages (9), and has been found to play a key role in pulmonary fibrotic disease by attracting immune cells and stimulating collagen overproduction (10, 11). Strikingly, elevated CCL18 in SSc sera are completely blocked by the anti-IL-6 receptor antagonist tocilizumab (4, 5), indicating that serum CCL18 is a biomarker for IL-6 activity in SSc. The distinct gene expression signature of SSc-ILD compared to healthy lungs also shows upregulation of macrophage marker genes, *AIF1*, *CD163*, *MS4A4A*, as well as secreted phosphoprotein I (*SPP1*) (8, 12), a marker of profibrotic macrophages in idiopathic pulmonary fibrosis (IPF) (13, 14). Lower lung lobes of IPF patients, where fibrosis is typically more advanced, demonstrated increased number of macrophages expressing *SPP1* compared to upper IPF lung lobes and controls (14). Additionally, in murine bleomycin-induced lung fibrosis, intravenous administration of Ly6C^{hi} inflammatory monocyte progenitor cells facilitates the progression of fibrosis through increase of alternatively activated lung macrophages, whereas macrophage depletion during fibrinogenesis is associated with less fibrosis (15).

In order to study the pathophysiology of SSc-ILD and define the role of profibrotic macrophages, we analyzed gene expression and altered chromatin structure of pulmonary macrophages from control and SSc-ILD lungs using single cell RNA sequencing (scRNA-seq) and single cell Assay for Transposase Accessible Chromatin sequencing (scATAC-seq). The conceptual basis for our work is that chromatin structure indicates the underlying transcriptional regulation. Chromatin structural changes or remodeling, where chromatin is open or accessible to transcription factors, is associated with regulated gene expression, affecting the transcriptome and consequently the cell phenotype (16). We sought to identify key transcription factors driving the altered transcriptome and differentiation of profibrotic lung macrophages, as well as to gain insight on signals, including cytokines, contributing to profibrotic macrophage differentiation.

METHODS

Single cell RNA-seq using 3' v3 and 5' v1 chemistries (10X Genomics) was performed on 11 explanted lung tissue samples, including both SSc-ILD and healthy control lungs (Table S1). Single Cell Reagents (10X Genomics, 3' v3 or 5' v1 chemistries) were used for the library preparation samples after digestion as described by Valenzi et al. (13). Library quantification and sequencing of the scRNA-seq cDNA libraries was carried out by the UPMC Genome Center, on an Illumina NextSeq-500 (14).

Healthy control and SSc-ILD lung samples were also analyzed for scATAC-seq (10X Genomics, Single Cell ATAC Reagent Kits, v1 Chemistry). Data were analyzed using the R packages Seurat v 4.0 (17) and Signac v 1.0.9004 (18), Loupe Browser 3.1.1 (10X Genomics) and Single-cell regulatory network inference and clustering (SCENIC) (19). Detailed methods are described in supplemental detailed methods file.

RESULTS

Profibrotic macrophages show discrete changes in transcriptomes

We analyzed lungs from six healthy control (HC) and five SSc-ILD patients by scRNA-seq (Table S1). We carried out bioinformatics analyses of samples in two groups because two different scRNA-seq chemistries were utilized, with different numbers of SSc-ILD versus healthy control lungs analyzed by each chemistry. Given the uneven number of sample types, batch correction between chemistries was problematic and might have obscured changes in macrophage transcriptomes associated with SSc-ILD. We first describe five lung samples including one healthy control and four SSc-ILD lungs, in which single cell cDNA libraries were prepared using 3' v3 Chemistry (10X Genomics). After dimensional reduction and visualization by Uniform Manifold Approximation and Projection (UMAP) (20), we identified cell types in each cluster as we have described previously (13), using characteristic gene markers for each cell population (Figure 1A, Figures S1–S3). Myeloid cells were identified by markers: *CD163*, *AIF1* and *MARCO*, and three macrophage subpopulations were identified by highly differentially expressed gene markers, similar to those seen in patients with idiopathic pulmonary fibrosis (13): SPP1 Mφ, Fatty Acid-Binding Protein 4 Mφ (FABP4, hereafter referred to as FABP4 Mφ) and Ficolin-1 Mφ (FCN1, hereafter referred to as FCN1 Mφ). The vast majority of the SPP1 Mφ cluster consisted of macrophages from the SSc-ILD patients, with few SPP1 Mφ from control lungs (Figures 1B, 1C). Notably, *SPP1* was upregulated in the SSc macrophages compared to normal macrophages within the same cluster (Figures S1–S4). Dendritic cells (DCs) were rare and clustered together with the *FCN1*-expressing Mφ (14), (Figure 1, Figure S4).

Comparing SPP1 Mφs to the FABP4 and FCN1 Mφs revealed multiple differentiatially expressed genes (Table S1), including *SPP1*, *Legumain* (*LGMN*), seen co-regulated in our previous analyses (14), and *Phospholipase A2 Group VII* (*PLA2G7*), a target of transcriptional regulation discussed below (Figure S4). Differentially expressed genes associated with each macrophage subpopulation compared to the other two subpopulations, were determined and queried for enriched gene ontology pathways. Upregulated genes in SPP1 Mφ compared to FABP4 Mφ and FCN1 Mφs revealed enrichment of genes

implicated in lipid metabolism and myeloid cell activation (Figures S5, S6, Table S2). Lipid metabolism plays a key role in macrophage activation by fatty acid synthesis to be utilized as precursors for inflammatory mediators synthesis with significant effect on the course of many metabolic diseases (21, 22). Upregulated differentially expressed genes in FABP4 Mφ compared to SPP1 Mφ and FCN1 Mφs demonstrated enrichment for negative regulation of macrophage differentiation, regulation of cholesterol storage and myeloid leukocyte activation pathways (23–25), (Table S3).

Lung myeloid cell subpopulations

We reclustered the myeloid cells from these samples, including macrophages, dendritic cells and proliferating Mφ guided by Clustree (Figure S7). This subclustering segregated most cells from the SSc and healthy samples into different clusters (Figure 2). The largest cluster, SSc-ILD SPP1 Mφ, selectively expressed *MER proto-oncogene*, *Tyrosine Kinase (MERTK)* and *LGMN*; SSc FABP4 Mφ selectively expressed *Inhibin Subunit Beta A* (*INHBA*); while HC and SSc FCN1 Mφ selectively expressed *Interleukin 1 Beta* (*IL1B*) as well as *Sialic Acid Binding Ig Like Lectin 10* (*SIGLEC10*) gene markers (Figure 2). These and other marker genes defined these macrophage subpopulations (Figure S4, Table S4).

These results mostly agree with our previous work examining macrophages in IPF (14). *Interferon Alpha Inducible Protein 27* (*IFI27*), an interferon regulated gene, and *CCL18*, a strong serum pharmacodynamic biomarker for tocilizumab (4, 5), were both more highly expressed in SSc SPP1- and FABP4-Mφ and the proliferating Mφ clusters compared to HC SPP1- and FABP4 Mφ (Figure 2). Although *IFI27* was also expressed by other lung cell populations, *CCL18* was almost exclusively expressed by lung macrophages. The proliferating Mφ cluster included both SPP1- and FABP4-Mφ, as this cluster showed a group of cells expressing *SPP1* (Figure 2C) and other markers of SPP1 cells (*MERTK* and *LGMN*, Figure 2D), and another group of cells expressing *FABP4* (Figure 2C) and other markers of FABP4 cells (*INHBA*, Figure 2D). These proliferating macrophages originated almost exclusively from the SSc-ILD patient lungs (Figure 2B), indicating that proliferating Mφ potentially differentiate into profibrotic SPP1 Mφ phenotype. The dendritic cells, also originated almost entirely from the SSc-ILD lungs, clustered into a discrete cluster expressing *CD1C* (Figure 2C), and other markers of cDC2 dendritic cells (not shown), (26).

Validation of macrophage subsets in SSc-ILD 5' v1 Chemistry

We extended the results above by analyzing scRNA-seq data from an additional five HC and one SSc-ILD lungs, in which single cell cDNA libraries were prepared using 5' v1 chemistry (10X Genomics). Myeloid subpopulations reflected those found in the analogous 3' v3 subclustering (Figure 3, Figures S8–S11). Of note, most of the macrophages in the SPP1 Mφ cluster originated from the SSc-ILD patient, consistent with the 3' v3 chemistry clustering. The proliferating Mφ population was relatively small as there was only one SSc-ILD lung and five HC lungs, however both *SPP1* and *FABP4* expression was noted (Figure S9). The proliferating cell cluster also included some control natural killer (NK) cells.

CCL18 (4, 5), *IFI27*, *Matrix Metallopeptidase 9* (MMP9), and *PLA2G7* (27) were upregulated in the SPP1 Mφ from the SSc-ILD lungs compared to SPP1 Mφ from HC lungs (Figure S9). Notably, *IFI27* gene expression in the serum of SSc patients is associated with digital ulcers (28). *MMP9* is also elevated in the serum of SSc patients and correlates with the Modified Rodnan Skin Score (MRSS) (29). In addition to *SPP1*, *PLA2G7*, discussed further below, differentiated SPP1 Mφ compared to FABP4 and FCN1 Mφ (Figures S12, S13).

Combined batch corrected datasets

In order to examine a larger dataset including both SSc-ILD and controls, we Harmony batch corrected and combined our two datasets (Figures S14–S20). Similar to our previous analyses, we identified the same cell types including myeloid cells after dimensional reduction and visualization by UMAP. Consistent with our previous results, the SPP1 Mφ cluster was formed mainly by Mφ from SSc-ILD patients. We subsequently reclustered the myeloid cells. As above, SPP1 macrophages, expressing also gene markers *MERTK*, *LGMN*, *CCL18*, *MMP9*, *TFEB*, *PLA2G7*, were almost exclusively from SSc-ILD samples. Thus, the results of this combined analysis are consistent with analyses of the uncombined datasets.

Pathway analysis of upstream regulators of SPP1 Mφ.

To addresss upstream drivers of macrophage differentiation in SSc-ILD, we analyzed genes differentially upregulated in SPP-1 Mφ using Ingenuity Pathway Analysis. This analysis supported several different mediators as potential upstream drivers of the SSc-ILD phenotype, including LPS and other TLR activators, IL-6 and TGF-β, all of which have been implicated in previous studies, as well as IL-33 and other cytokines (Table S5)

Skin microarray data suggest similar pathologic changes occur in other SSc organs

Our data examining the epigenetics of the profibrotic macrophages in SSc-ILD may also be relevant for skin fibrosis and other target organs in SSc. IL-6 inhibition with tocilizumab in the faSScinate clinical trial, while just missing a statistically significant p-value in both phase 2 and phase 3 trials, downregulated genes associated with M2 macrophages (30), and decreased expression of TGF-β regulated genes in explant fibroblast cultures (31). In order to better understand whether SPP1 macrophages contribute to fibrosis in other SSc organs, we examined gene expression in SSc skin. *SPP1* and *CCL18* expression was markedly increased in bulk SSc skin RNA. Previously described skin scRNA-seq data indicated that the expression of these genes comes primarily from myeloid cells in the skin (Figures S21–S23).

Prediction of transcription factors ATF5 and TFEB regulating SPP1 macrophage differentiation

Single-cell regulatory network inference and clustering (SCENIC) provides a bioinformatics method for reconstructing gene regulatory networks, as well as identifying cell states, providing insights into the transcription factors leading to cellular heterogeneity (19). SCENIC identifies regulons, a transcription factor and its associated downstream target

genes, by examining coregulated gene expression in a single cell dataset and using RcisTarget to confirm transcription factor binding sites in downstream target genes. We used SCENIC to predict regulons and transcription factors regulating SPP1 Mφ differentiation, comparing the transcriptomes of SSc-ILD SPP1 Mφ to all FABP4 Mφ, all FCN1 Mφ and proliferating Mφ. These cells were then clustered by their regulon activity score, and labeled according to the cell population, patient individual identity and health status (Figure 4A). Notably, SPP1 Mφ formed the biggest cluster, composed almost entirely of cells from SSc-ILD lungs and included cells from each SSc-ILD sample. SPP1 Mφ were further examined by SCENIC for regulons predicited to selectively regulate the SSc SPP1 Mφ transcriptome (Figure 4B–D). Multiple regulons (Figure 4D) and their associated transcription factors (Figure 4C) were selectively upregulated in SPP1 Mφ compared to the other macrophage populations. Most of these regulons were also upregulated in the proliferating macrophage population, of which most of the cells expressed *SPP1* and are thus likely contributing to the SSc SPP1 Mφ population. The regulons for Activating transcription factor 5 (ATF5) and Transcription factor EB (TFEB) both have *SPP1* and *MMP9* as target genes (Figures S24, S25). *SPP1* and *MMP9* are not only marker genes of the SPP1 Mφ population, but also have profibrotic activity (12, 14, 29). *SPP1* gene expression is upregulated in SSc-ILD and IPF lungs compared to control lungs based on findings from our previous work as well as from other groups (12, 14). *SPP1* is included in the differentiating genes between SSc-ILD and IPF lungs in comparison to control lungs (12). Increased serum MMP9 correlates with the degree of skin fibrosis in SSc patients through fibroblast activation and acceleration of fibrosis under the effect of proinflammatory cytokines such as tumor necrosis factor β (TGF-β) and interleukin 1β (IL-1β) (29). Thus SCENIC particularly implicates TFEB and ATF5 as key transcription factors regulating SPP1 Mφ cell differentiation, likely promoting the profibrotic activity of these cells.

Altered chromatin structure implicates transcription factors regulating pro-fibrotic SSc-ILD SPP1 macrophage differentiation

In order to better understand the transcription factors regulating differentiation of SSc-ILD SPP1 Mφ, using an orthogonal technology to SCENIC predictions, we analyzed lung samples by scATAC-seq in parallel with scRNA-seq (Table S1). ScATAC-seq examines the chromatin structure of nuclei from each cell by cleaving open chromatin with a Tn5 transposase, and then partitioning cells and ligating cell specific bar-codes to the DNA fragments. The resulting fragments indicate open chromatin structure on a cell-by-cell basis. We analyzed scATAC-seq by clustering nuclei for both SSc-ILD samples using two bioinformatics approaches: Loupe software (10X Genomics) and Signac (18), (Figures S26–S28). For Loupe, the cell types in each cluster were identified by examining the accessibility of promoters of gene markers for each cell type. Macrophages were identified as having open chromatin for the promoters of *CD163*, *AIF1* and *MARCO*, and FABP4 and SPP1 Mφ populations identified by open chromatin in the respective 5' promoters (Figure S27). In a complementary analysis we identified nuclei clusters by integrating with the paired scRNA-seq data to predicted cell types (Signac) (18). Signac software maps the cells by examining open chromatin in promoters of differentially expressed genes defined by scRNA-seq cell clusters (Figures S27, S28). We used both Loupe and Signac to determine enriched, accessible motifs for transcription factors, reflecting open chromatin across the

genome, comparing SPP1 Mφ to FABP4 Mφ for each SSc-ILD sample (Table S1, Figures S25, S29). Both of these programs use the Jaspar database to identify transcription factor binding motifs (32).

Strikingly, many of the transcription factors binding motifs enriched in open chromatin of SPP1 Mφ were the same transcription factor associated regulons predicted by SCENIC to regulate the differentiation of profibrotic SPP1 Mφs: TFEB, Activating Transcription factor 6 (ATF6), Microphthalmia-associated transcription factor (MITF), Basic Helix-Loop-Helix Family member E40 (BHLHE40), Kruppel-Like Factor 6 (KLF6), ETS Variant Transcription Factor 5 (ETV5), Nuclear Receptor Subfamily 1 Group H Member 3 (NR1H3), CAMP Responsive Element Binding Protein 3 Like 2 (CREB3L2), MAF BZIP Transcription Factor B (MAFB), and AT-Rich Interaction Domain 3A (ARID3A) (Table 1 and Figure 4C). In addition, multiple members of the activating protein-1 (AP-1) transcription factor family, binding as homo and heterodimers, were identified across both software outputs. AP-1 proteins include the JUN, FOS, ATF and musculoaponeurotic fibrosarcoma (MAF) protein families (33). Specifically, the JUN family proteins c-JUN, JUNB, JUND, FOS family proteins c-FOS (FOS), FOSB, FRA1 (FOSL1), FRA2 (FOSL2), and MAF family proteins c-MAF (MAF), MAFA, MAFB, MAFF, MAFG and MAFK were enriched. Of these, the MAF regulon was also predicted by SCENIC to regulate SPP1 Mφ differentiation (Figure 4D).

As examples of the epigenetic modifications associated with differentiation into SPP1 macrophages, we examined accessible chromatin of selected marker genes: *SPP1*, *MMP9* and *FABP4*, comparing SPP1 Mφ and FABP4 Mφ in SSc-ILD (Figure 5, Figures S30–S32). The *SPP1* gene showed more accessible chromatin for the SSc-ILD SPP1 Mφ compared to FABP4 Mφ in the promoter region immediately proximal to the transcriptional start site, as well as regions further upstream of the promoter and the 4th intronic region (Figure 5). The *MMP9* gene also showed more accessible chromatin in SPP1 Mφ, but in this case increased accessiblity was not seen at the promoter but rather in regions around exon 6, exons 9–12 and introns (Figure 5). In contrast, the *FABP4* gene showed strikingly more accessible chromatin in a region proximal to the transcriptional start site and in a broad second region 3' from the gene in FABP4 Mφ compared to SPP1 Mφ (Figure 5).

Although we also examined scATAC-seq from several control lungs, technical variability and the inability to clearly distinguish in the scATAC-seq assay between macrophage populations precluded direct comparison of SSc-ILD and control macrophages (Figures S33–S37).

Increased SPP1/MERTK Mφ and TFEB expression in SSc-ILD.

Finally, in order to show increased SPP1 Mφ in SSc-ILD, we identified these cells by staining the co-expressed surface marker MERTK (Figure S38). In addition, we found that a subpoplation of these cells strongly co-expressed TFEB (Figures S39–S40). Available antibodies did not allow us to detect ATF5.

DISCUSSION

The mechanisms driving fibrosis in SSc-ILD are currently uncertain, however the association of macrophage gene expression, particularly of *SPP1*, with progressive fibrosis makes these cells likely mediators of profibrotic signals to fibroblasts (8). This study confirms the highly increased numbers of a subpopulation of macrophages, characterized by upregulated expression of *SPP1* and *MMP9*, as well as a broader transcriptome in SSc-ILD, in agreement with previous studies both of our group and others (9–16). Analysis of the differentially expressed genes between SPP1 Mφ compared to all other Mφ subpopulations linked SPP1 Mφ to lipid metabolism pathways. Lipid metabolism plays an important role in macrophage function (21). Several transcription factors, including Peroxisome proliferator-activated family of receptors (PPARs) and CCAAT enhancer binding proteins (SREBPs), have been implicated in lipid metabolism in macrophages (21). Further investigation of the effect of lipid metabolism on the development of Mφ phenotype would be important, in view of drugs targeting metabolic pathways (21–25).

SPP1 and *MMP9* genes were selectively upregulated by SSc SPP1 Mφ. Osteopontin (OPN), the product of SPP1, is increased in the serum of patients with systemic sclerosis (34, 35) and has been implicated in idiopathic pulmonary fibrosis (36), as well as renal and bone marrow fibrosis (37, 38), suggesting that SPP1 Mφ may have a more general role in promoting fibrosis. *MMP9* gene expression is also elevated in idiopathic pulmonary fibrosis (39). Additionally, MMP9 activity is increased in bronchoalveolar lavage fluid of rapidly progressive IPF and in SSc-ILD (40). The expression of *MMP-9* in SSc-ILD lungs uniquely by SPP1 Mφ and proliferating SPP1 Mφ suggests that increased levels in serum and BAL in SSc-ILD patients may reflect the activity and/or degree of lung infiltration by these cells, and thus might serve as a biomarker for their activity (35).

We used two approaches to definine likely transcription factors controlling the differentiation of SPP1 Mφ: SCENIC analysis of the SPP1 Mφ transcriptome and scATAC-seq analysis of open chromatin in SPP1 Mφ. SCENIC analyzes gene regulatory networks, i.e., it looks at genes co-regulated with transcriptions factors of each cell. Several approaches exist for identifying target genes directly regulated by transcription factors, (41), including: 1) direct TF-DNA binding assays; 2) computational predictions of transcription factor–target interactions, i.e., position weight matrices (PWMs); and 3) gene regulatory networks that assume transcription factor expression levels regulate targeted genes. SCENIC uses approach 3) in its initial screening, with the resulting gene regulatory network then filtered with CisTarget, a database incorporating results used in methods 1 and 2 (42). The concordance between the predictions from SCENIC with our scATAC-seq data are mutually reinforcing, but in particular strongly reinforce the value of SCENIC in analysing scRNA-seq transcriptomes.

Our scATAC-seq data show characteristic chromatin pattern changes for SPP1 Mφ with increased accessibility of SPP1 and MMP9, and decreased accessibility of FABP4 reflecting the altered binding of transcription factors and enhancers comparing SPP1 Mφ to FABP4 Mφ. These observations provided confidence in our scATAC-seq data. However, altered open chromatin peaks are too broad to implicate specific transcription factors on the basis

of individual genes, and thus Signac bioinformatics, examining transcription factor binding sites in open chromatin across the genome provided a more robust method for detecting the likely role of specific transcription factors regulating the phenotype of SPP1 Mφ. This identified numerous transcription factors, including ATF5, TFEB, BCL11A, ETV5, JUN, and others, requiring further experimental validation for their specific roles in regulating SPP1 Mφ differentiation.

Several of the transcription factors identified to participate in SPP1 Mφ differentiation have been implicated in profibrotic phenotypes in other settings. TFEB potentially plays role in silicosis, an occupational irreversible fibrotic lung disease, through disruption of lysosomal autophagy in alveolar macrophages (43). In particular, pulmonary fibrosis in silicosis is promoted with phagocytosis of crystalline silica (CS) particles by alveolar macrophages resulting in apoptosis and inflammation. There is increasing evidence of TFEB interplay with TGF-β (44). TGF-β inhibition associated with blockade of BRAF inhibition and TFEB phosphorylation contributes to malignant cells responsiveness to chemotherapy in melanoma (45). Thus, the effect of TFEB on profibrotic SPP1 Mφ differentiation requires further study. ATF6 and its role in macrophage endoplasmic reticulum stress has also been implicated in the pathophysiology of lung fibrosis (46). Similarly, Sterol Regulatory Element Binding Transcription Factor 1 (SREBF1) was implicated in progression of murine pulmonary fibrosis (47), and BHLHE40 has been identified as an important homeostatic regulator of macrophages in lungs through pulmonary surfactant turnover (48).

Members of the AP-1 transcription factor family, predicted in our analysis to play an important role in the profibrotic phenotype of SPP1 Mφ, have been investigated in the pathophysiology of many fibrotic diseases. As the binding sites for these family members are similar, our data do not clearly discriminate which family member is most important in SPP1 Mφ differentitation. Overexpression of JUN, a prototype AP-1 family member, is associated with severe multi-organ fibrosis in murine models (49). Fos-related antigen-2 transcription factor (Fra-2 or FOSL2) overexpression has been associated with murine SSc-like lung fibrosis (50). Myofibroblasts are activated in vitro by macrophages in a Fra-2-dependent manner, consequently promoting fibrosis. In addition, both myeloid cell inactivation of Fra-2 and Fra-2/AP-1 inhibitors attenuate pulmonary fibrosis in murine bleomycin models (50). Thus, our data strongly support these murine studies, indicating a critical role for AP-1 transcription factors in the development of profibrotic SPP1 macrophages in human SSc-ILD. Defining the specific AP-1 family member(s) most important in regulating SSc-ILD SPP1 Mφ will be key for potential targeting of Fra-2 or other AP-1 family members in SSc-ILD.

Proliferating cells in our dataset were largely composed of proliferating SPP1 and FABP4 Mφ from SSc-ILD lungs, consistent with our previous work showing proliferating macrophages in idiopathic pulmonary fibrosis (14). While the stimulus for proliferation of these cells is uncertain, regeneration of tissue resident Mφ through M-CSF and Granulocyte-Macrophage colony-stimulating factor (GM-CSF) is known to occur in repopulation of inflamed tissues following resolution of inflammation (51). IPF scRNA-seq also indicates increased *CSF1* expression in lung mast cells and upregulation of *LGMN* and other genes regulated by IL-4 (14, 52). *CSF1* is upregulated by SSc-ILD SPP1 Mφ and mast cells, as

well as by control pericytes, smooth muscle cells and fibroblasts. These findings support proliferating macrophages as the primary source for profibrotic SPP1, suggesting that antiproliferative agents or drugs blocking macrophage cytokines might have efficacy in treating SSc-ILD.

There is increasing evidence linking chemokine CCL18 with pulmonary inflammation, supporting its potential future use as early marker of SSc-ILD (9, 30, 35, 53). Our data reflected upregulation of *CCL18* in SPP1 Mφ from the SSc-ILD lungs compared to HC. Elevated CCL18 in the serum and supernatants of cultured bronchoalveolar lavage (BAL) of SSc patients is associated with more advanced ILD by pulmonary function testing (53). CCL18 concentration in the serum of SSc patients was strikingly decreased following treatment with tocilizumab and *CCL18* gene expression in skin biopsies of SSc patients was downregulated following tocilizumab treatment, along with other macrophages markers (30). In light of the recent FDA approval of tocilizumab for decreasing progression of SSc-ILD, *CCL18* has emerged as a marker of IL-6 in SSc-ILD. By extention, the upregulation of *CCL18* in SSc-ILD SPP1 Mφ compared to HC seen on our results supports the role of SPP1 Mφ as drivers of fibrosis in SSc-ILD. Possibly, *CCL18* and *SPP1* expression characterize macrophages with common functional background and profibrotic potential (35).

The limitations of this study include preclusion of direct comparison of SSc-ILD and control macrophages with scATAC-seq due to technical variability and bioinformatics challenges to clearly distinguish macrophage subpopulations after scATAC-seq in normal lungs. Additionally, since the lung explants are obtained at the time of lung transplant indicating advanced stage of SSc-ILD, they may reflect later stages of SSc-ILD. It is possible that earlier stages of SSc-ILD might have different mechanisms driving disease. Furthermore, it is noted that sample size is small, scRNA-seq analysis groups 3' and 5' are skewed respectively towards SSc-ILD vs HC lungs and that single-cell analyses, including data integration, is parameterizable.

In summary, our transcriptomal analysis demonstrates the transcriptome of profibrotic SPP1 Mφ in SSc-ILD and uncovers critical transcription factors in the profibrotic phenotype through changes in chromatin accessibility. These findings support further investigation of key transcription factors, particularly of ATF5 and TFEB, that activate SPP1 Mφ signature genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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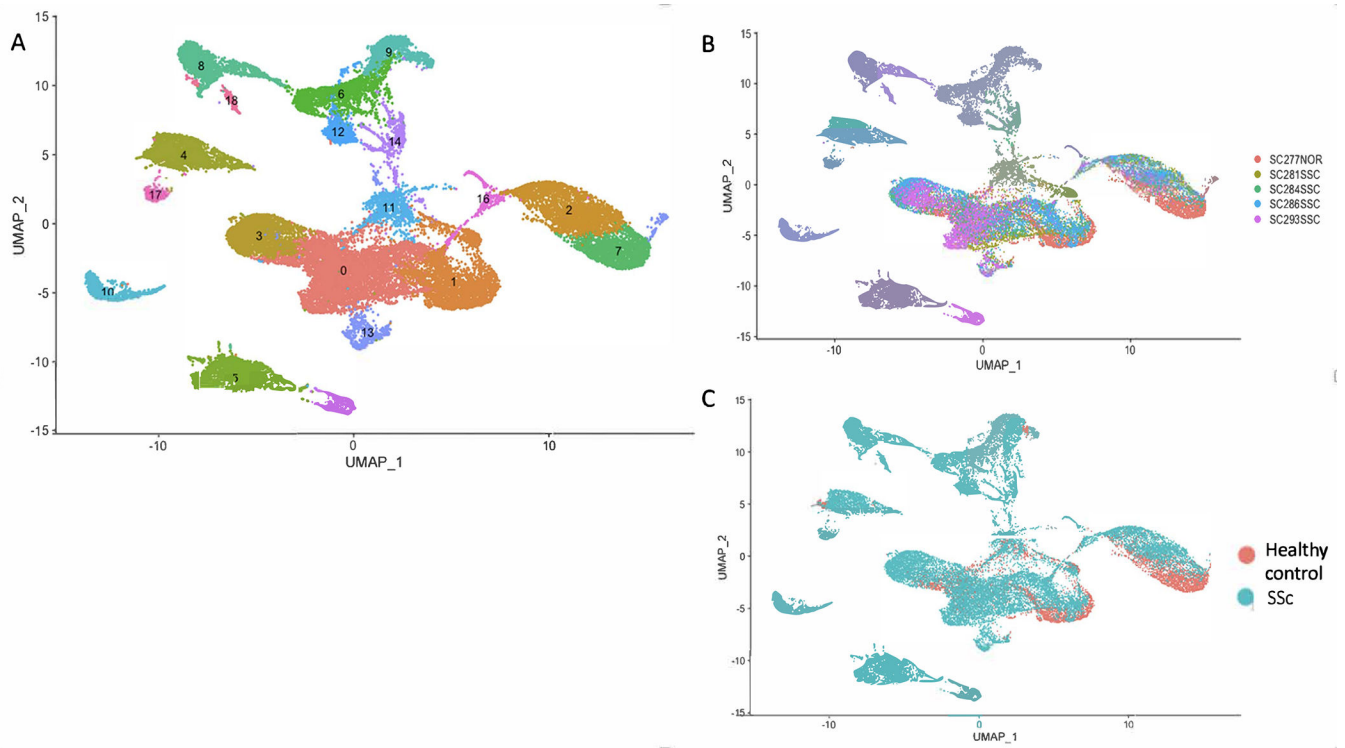
Figure 1.
Single-cell RNA-sequencing analysis of 5 lung samples in 3' v3 chemistry. (A) Visualization of clustering by UMAP per cell type: 0-SPP1 macrophages, 1-FCN1 macrophages and Dendritic cells, 2-T cells, 3-FABP4 macrophages, 4-Endothelial cells, 5-Fibroblasts, 6-Goblet cells and Alveolar Type 1 cells, 7-NK cells, 8-Ciliated cells and Goblet cells, 9-Alveolar Type 2 cells, 10-Mast cells, 11-Low quality cells, 12-Basal cells, 13-Proliferating cells, 14-Goblet cells, 15-Pericytes and Smooth muscle cells, 16-B cells, 17-Lymphatic endothelial cells, 18- Ciliated cells. Macrophage subpopulations, dendritic cells and proliferating cells are located along the center of the UMAPs. (B) Visualization of clustering by UMAP per individual identity. (C) Visualization of clustering by UMAP per health status. SPP1 macrophages cluster 0 is formed primarily of macrophages from the SSc-ILD patients.
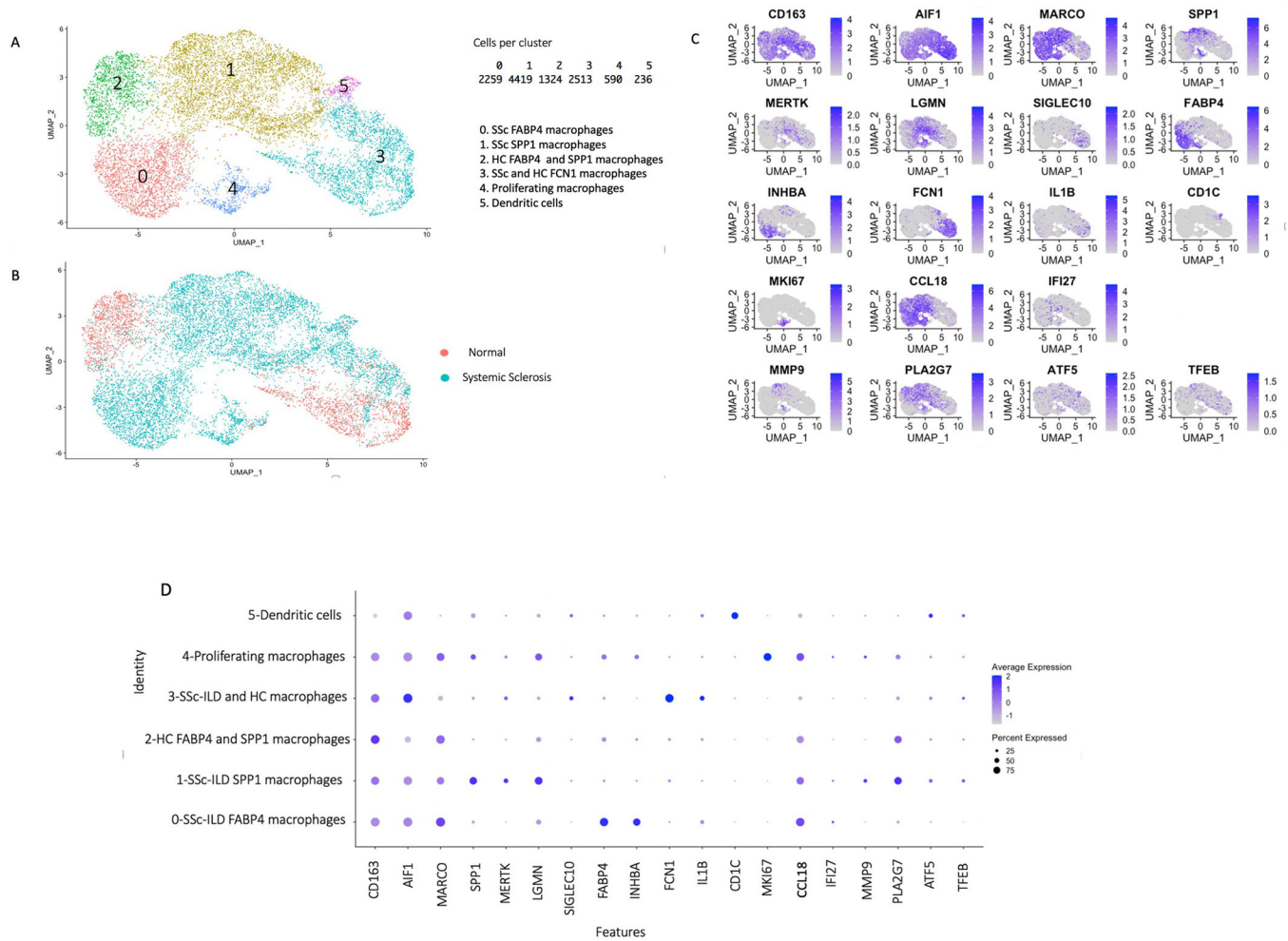
Figure 2.
Single-cell RNA-sequencing analysis of macrophage subpopulations, dendritic cells, proliferating Mφ from 5 lung samples in 3' v3 chemistry, 1 healthy control and 4 SSc-ILD lung samples. (A) Visualization of clustering by UMAP per cell type. (B) Visualization of clustering by UMAP per health status. (C) Visualization of gene expression by FeaturePlot of macrophage subpopulations gene markers, dendritic cells, proliferating Mφ and other genes expression during analysis of only macrophages, dendritic cells, proliferating Mφ. (D) Visualization of gene expression by DotPlot of macrophages, dendritic cells, proliferating Mφ from 1 healthy control and 4 SSc-ILD lung samples in scRNA-seq 3' v3 Chemistry. DotPlot shows upregulation of *SPP1, MERTK, LGMN* genes in SSc-ILD SPP1 Mφ and proliferating Mφ. *MMP9* and *PLA2G7* genes markers are upregulated in SSc-ILD SPP1 Mφ compared to all other macrophages.
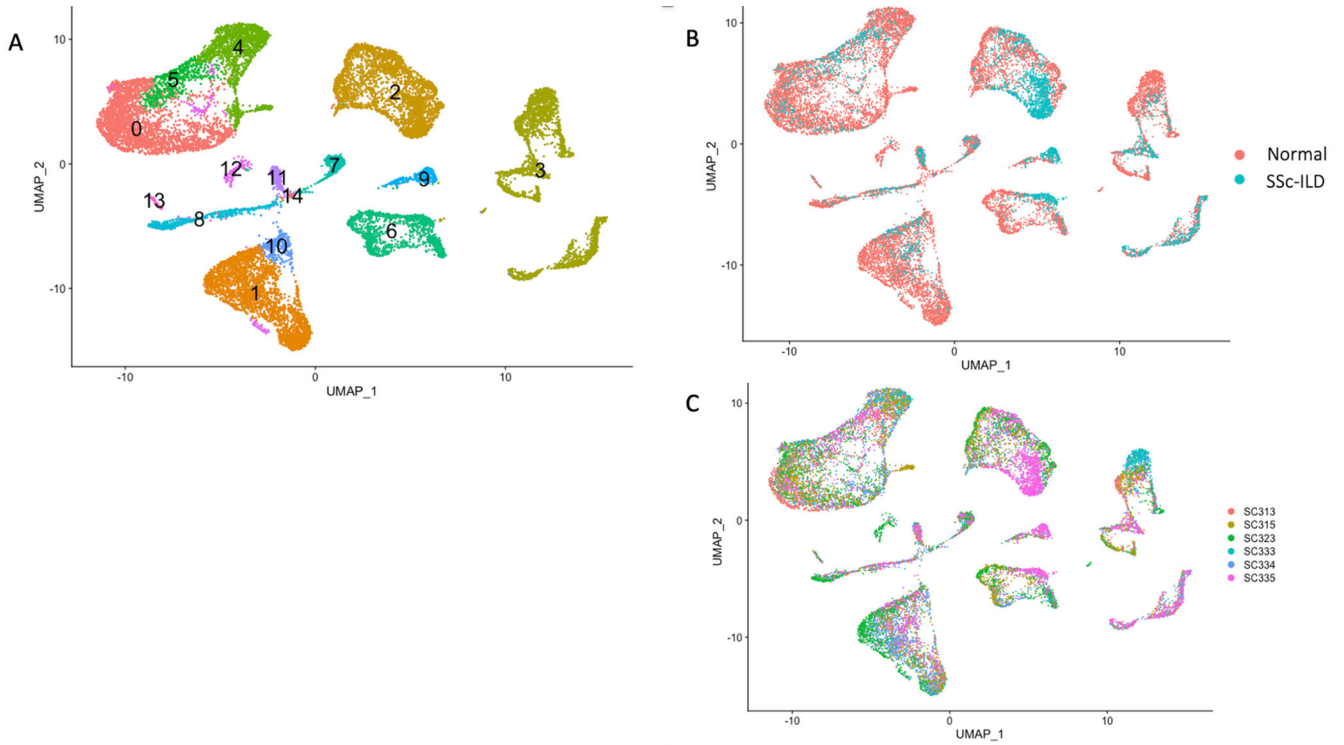
Figure 3.
Single-cell RNA-sequencing analysis of 6 lung samples in 5' v1 chemistry, including 1 SSc-ILD and 5 healthy control lungs. (A) Visualization of clustering by UMAP per cell type. Macrophage subpopulations, dendritic cells and proliferating cells are located along the left upper part of the UMAPs. Cell clusters were identified as follows: 0-FCN1 macrophages and Dendritic cells, 1-T cells and NK cells, 2-Endothelial cells, 3-Epithelial cells, 4-FABP4 macrophages, 5-SPP1 macrophages, 6-Fibroblasts, 7-Lymphatic endothelial cells, 8-B cells, 9-Pericytes and Smooth muscle cells, 10-Mitochondrial related genes, 11-Mast cells, 12-Proliferating cells, 13-Dendritic cells, 14-Hemoglobin related cells. (B) Visualization of clustering by UMAP per health status. SPP1 macrophages cluster 5 is formed primarily of macrophages from the SSc-ILD patient SC335. (C) Visualization of clustering by UMAP per individual identity.
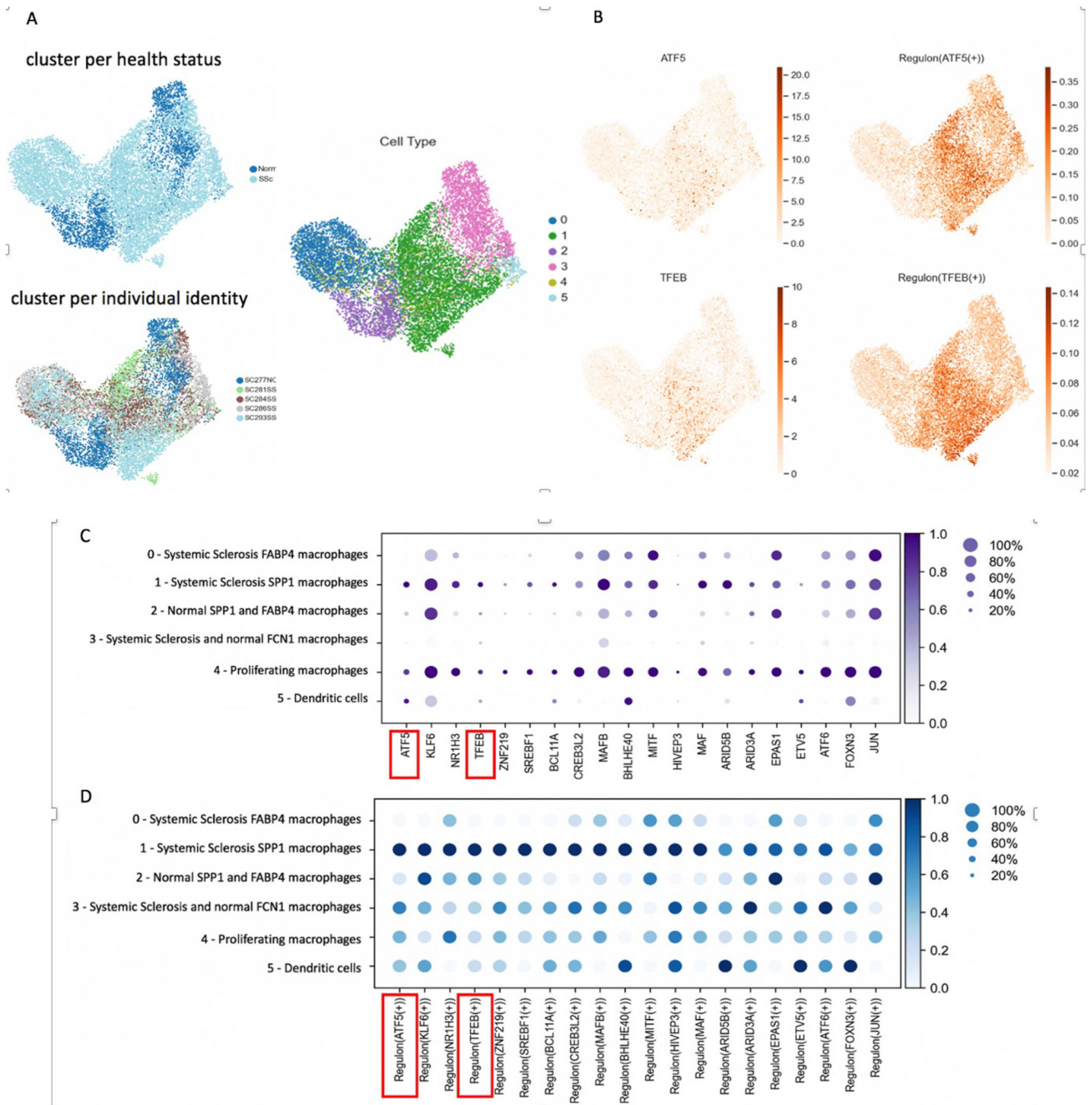
Figure 4.

Regulons and Transcription Factors predicted to be important for SPP1 Mφ during analysis of macrophages, dendritic cells, proliferating Mφ from 5 lung samples in scRNA-seq 3' v3 chemistry by SCENIC. (A) Cell clusters are identified as follows: 0- SSc-ILD FABP4 Mφ (blue), 1- SSc-ILD SPP1 Mφ (green), 2- HC SPP1 and FABP4 Mφ (purple), 3- SSc-ILD and HC FCN1 Mφ (pink), 4- Proliferating Mφ (yellow), 5- Dendritic cells (light blue). (B) *SPP1* is a target gene for ATF5 and TFEB transcription factors and regulons. (C) Transcription factor DotPlot predicted to be important for SPP1 Mφ by SCENIC including transcription

factors ATF5 and TFEB indicated in red box. (D) Regulon DotPlot predicted to be important for SPP1 Mφ by SCENIC including regulons ATF5 and TFEB indicated in red box.
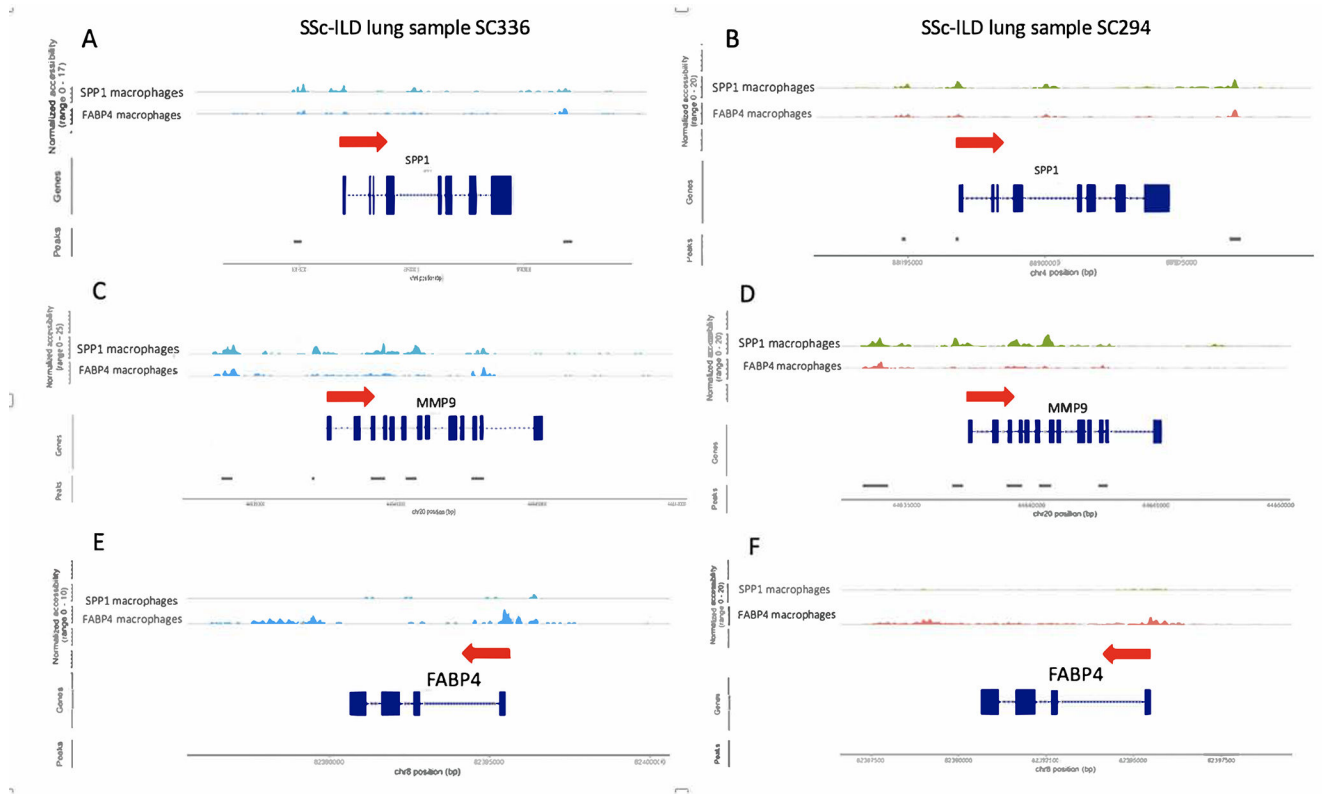
Figure 5.
ScATAC-sequencing analysis of 2 SSc-ILD lung samples by Signac showing chromatin
pattern changes for SPP1 macrophages compared to FABP4 macrophages. The red arrow
indicates the direction of transcription. Exons are shown in blocks and introns flank exons.
(A, B) *SPP1* gene showed more accessible chromatin for the SSc-ILD SPP1 Mφ compared
to FABP4 Mφ in the region proximal to the transcriptional start site for a SSc-ILD lung,
as well as regions further 5' of the promoter and intron 4. (C, D) *MMP9* gene also
showed more accessible chromatin in SPP1 Mφ, but in this case increased accessibility was
not seen around the promoter but rather in regions around exon 6, exons 9–12 and introns.
(E, F) *FABP4* gene showed strikingly more accessible chromatin in a region proximal to
the transcriptional start site and in a broad second region 3' from the gene in FABP4 Mφ
compared to SPP1 Mφ.

Table 1.

Accessible motifs to transcription factors for SPP1 macrophages from single-cell ATAC sequencing analysis of SSc-ILD lung samples. Enriched, accessible motifs to transcription factors for SPP1 macrophages compared to FABP4 macrophages as predicted by Signac and SCENIC software for SSc-ILD samples SC336 and SC294, including, respectively: MITF, TFEB, ATF6, SREBF1, BHLHE40, KLF6 and ETV5; MITF, SREBF1, TFEB, BHLHE40 and ETV5 as indicated in red boxes. Also indicated are AP-1 transcription family members including FOS, FOSL1, JUND, JUNB, BATF3, FOSL2, JUN, MAF, NFE2.

Motif Name	Fold enrichment	p value
SSc-ILD sample SC336		
FOS	3.19010822	6.86E-145
FOSL1::JUNB	3.4413536	1.02E-143
BATF3	2.9786597	2.52E-140
FOSL2: :JUN	3.32140474	5.48E-131
JUN(var.2)	3.04331528	1.07E-128
FOSL1::JUND	3.26643938	1.59E-127
FOSL2: :JUNB	3.23793531	9.57E-126
MAF::NFE2	2.66199575	5.99E-56
MITF	1.82166811	1.01E-05
TFEB	1.73941338	3.75E-05
ATF6	1.70764316	0.00072468
SREBF1	1.43184691	0.0011076
BHLHE40	1.09720706	0.36788806
KLF6	0.90018323	0.88278759
ETV5	0.71966307	0.99424075
SSc-ILD sample SC294		
NFKB2	3.01107011	0.00589081
RELA	2.74533414	0.00589081
NFKB1	2.70067517	0.00589081
FOSB::JUNB	2.65102267	0.00589081
FOSL2::JUND	2.63504611	0.00589081
JDP2	2.61442107	0.00589081
NFE2	2.52034217	0.00589081
JUN(var.2)	2.42862887	0.00589081
MITF	2.19428571	0.00589081
SREBF1	1.58342582	0.00589081
TFEB	1.81588903	0.00589081
BHLHE40	1.65048544	0.00589081
ETV5	0.62227754	0.00589081